Protoporphyrinogen oxidase-inhibiting activity of the new, wheat-selective isoindoldione herbicide, cinidon-ethyl

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Abstract: Cinidon-ethyl (BAS 615H) is a new herbicide of isoindoldione structure which selectively controls a wide spectrum of broadleaf weeds in cereals. The uptake, translocation, metabolism and mode of action of cinidon-ethyl were investigated in *Galium aparine* L, *Solanum nigrum* L and the tolerant crop species wheat (*Triticum aestivum* L). When plants at the second-leaf stage were foliarly treated with cinidon-ethyl equivalent to a field rate of 50 g ha^{-1} for 48 h, the light requirement for phytotoxicity and the symptoms of plant damage in the weed species, including rapid chlorophyll bleaching, desiccation and necrosis of the green tissues, were identical to those of inhibitors of porphyrin synthesis, such as acifluorfen-methyl. The selectivity of cinidon-ethyl between wheat and the weed species has been quantified as approximately 500-fold. Cinidon-ethyl strongly inhibited protoporphyrinogen oxidase (Protox) activity *in vitro*, with I_{50} values of approximately 1 nM for the enzyme isolated from the weed species and from wheat. However, subsequent effects of herbicide action, with accumulation of protoporphyrin IX, light-dependent formation of 1-aminocyclopropane-1-carboxylic acid-derived ethylene, ethane evolution and desiccation of the green tissue, were induced by cinidon-ethyl only in the weed species. After foliar application of [^{14}C] cinidon-ethyl, the herbicide, due to its lipophilic nature, was rapidly adsorbed by the epicuticular wax layer of the leaf surface before it penetrated into the leaf tissue more slowly. No significant differences between foliar and root absorption and translocation of the herbicide by *S nigrum, G aparine* and wheat were found. After foliar or root application of [^{14}C]- cinidon-ethyl, translocation of ^{14}C into untreated plant parts was minimal, as demonstrated by combustion analysis and autoradiography. Metabolism of [^{14}C]cinidon-ethyl via its *E*-isomer and acid to further metabolites was more rapid in wheat than in *S nigrum* and *G aparine*. After 32 h of foliar treatment with 50 g ha^{-1} of the [^{14}C]-herbicide, approximately 47%, 36%, and 12% of the absorbed radioactivity, respectively, were found as unchanged parent or its biologically low active *E*-isomer and acid in the leaf tissue of *G aparine, S nigrum* and wheat. In conclusion, cinidon-ethyl is a Protox-inhibiting, peroxidizing herbicide which is effective through contact action in the green tissue of sensitive weed species. It is suggested that a more rapid metabolism, coupled with moderate leaf absorption, contribute to the tolerance of wheat to cinidon-ethyl.

Keywords: Cinidon-ethyl; ethane; ethylene; *Galium aparine*; herbicide metabolism; uptake and translocation; protoporphyrinogen oxidase; protoporphyrin IX; *Solanum nigrum*; *Triticum aestivum*

1 INTRODUCTION

Herbicides which inhibit protoporphyrinogen IX oxidase (EC 1.3.3.4.; Protox), a key enzyme in tetrapyrrole biosynthesis, have been used for more than 30 years in agriculture.[1–3] Protox catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (Proto).[4] Blockage of the pathway at this enzymatic site prevents the synthesis of chlorophylls, hemes and cytochromes which are components of many important proteins involved in light harvesting, energy transduction, signal transduction and detoxification.[4] As a further consequence, plants treated with these herbicides accumulate high concentrations of Proto in the green tissues resulting from an extraplastidic oxidation of protoporphyrinogen IX.[1–3,5,6] Proto is known as a potent photosensitizer which is the primary photodynamic pigment responsible for the herbicidal activity. In the presence of light and molecular oxygen, Proto generates high levels of singlet oxygen and toxic oxygen radicals which attack the unsaturated fatty acids of the cell membranes.[1–3] Lipid peroxidation leads to membrane degradation, particularly of the plasmalemma, tonoplast and chloroplast envelope.[1,3] This causes ion leakage and water loss from the cell. Further effects are inhibition of photosynthesis, formation of 1-aminocyclopropane-1-carboxylic acid (ACC)-dependent stress ethylene, evolution of ethane and malondialdehyde which are end-products of membrane peroxidation, and finally bleaching of chloroplast pigments and cellular death.

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The green tissues become necrotic and brown and damaged plants die a few days after treatment.[1,3]

The most important chemical classes of commercial and experimental Protox-inhibiting herbicides include *p*-nitrodiphenyl ethers, phenylpyrazole ethers, pyridine carboxamides, *N*-aryl-substituted pyrazoles and triazolinones, and substituted aryl derivatives nitrogen-linked to a heterocycle such as an oxadiazole or isoindoldione.[3,7] Cinidon-ethyl is a new isoindoldione herbicide which has been introduced commercially in Europe in 1999. When applied post-emergence, the compound selectively controls a wide spectrum of broadleaf weeds, including *Galium aparine* L, in cereals.[8] After foliar treatment, cinidon-ethyl elicits the same type of phytotoxic symptoms, with yellowing and necrosis, as Protox-inhibiting, peroxidizing herbicides in susceptible plant species.

The aims of the present study were to elucidate the biochemical mode of action and the mechanism of selectivity of cinidon-ethyl between *G aparine*, *S nigrum* and the crop species wheat by directly comparing the uptake, translocation and metabolism of the herbicide and the effects on Protox activity, Proto accumulation, ethane and ACC-derived ethylene formation and leaf fresh weight.

2 MATERIALS AND METHODS

2.1 Chemicals

Cinidon-ethyl [ethyl (*Z*)-2-chloro-3-[2-chloro-5-(1,3-dioxo-4,5,6,7-tetrahydroisoindol-2-yl)phenyl] acrylate; BAS 615H; Fig 1), [*U-phenyl*-^{14}C]cinidon-ethyl (>98% radiochemical purity, sp act 2187 MBq mmol^{-1}, 5.55 MBq mg^{-1},), a 200 g litre^{-1} cinidon-ethyl EC (BAS 615 00H) containing a castor oil ethoxylate emulsifier, the plant metabolites of cinidon-ethyl, BAS 615M01 (the 'acid' of cinidon-ethyl, (*Z*)-2-chloro-3-[2-chloro-5-(1,3-dioxo-4,5,6,7-tetrahydro-isoindol-2-yl) phenyl]acrylic acid) and BAS 615M00 (*E*-isomer of cinidon-ethyl), and acifluorfen-methyl were from BASF Aktiengesellschaft, Ludwigshafen, Germany.

2.2 Cultivation of plants

Seeds of *Galium aparine* L and *Solanum nigrum* L were germinated in soil in the greenhouse. Seedlings were transferred to vermiculite moistened with half-strength Linsmaier–Skoog nutrient solution[9] and cultivated under controlled conditions in a climate room (photon irradiance *c* 250 μmol m^{-2}s^{-1}, 400–700 nm, fluorescent lamps, Radium HRLV, 1000 W; 16:8 h light:dark cycles at 25/20 °C). Young plants of *Triticum aestivum* L cv Kanzler were raised in vermiculite substrate. For cultivation of plants in hydroponics, uniformly developed plants were transferred into 300-ml glass vessels with half-strength Linsmaier–Skoog medium and cultivated in growth chambers (light: 530 μmol m^{-2}s^{-1}, 400–750 nm, Osram powerstar HQI-R 250 W/NDL and Osram krypton 100 W lamps) at 22 °C and 75% relative humidity (*S nigrum, G aparine*: three plants per vessel, wheat: six plants per vessel; five replicates). The solution was aerated throughout the experiments. Plants were treated after one day of adaptation.

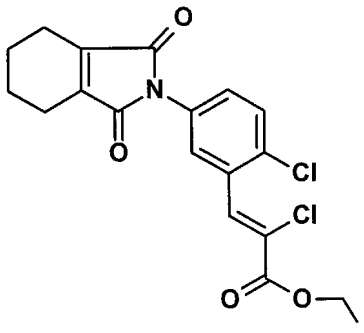

Figure 1. Structural formula of cinidon-ethyl.

2.3 Ethylene, ethane, ACC and Proto levels in leaf discs

Discs (0.4 cm diameter) were cut from blades of the second and third leaves (whorl) of plants with a corkborer under dim, green light conditions. Discs were floated for 1–2 h in double-distilled water so that ethylene produced from the excision process could dissipate.[10] Twenty randomized leaf discs were then placed adaxial side down on a filter paper in a Petri dish (5 cm diameter, six replicates) moistened with 1 ml MES (2-[*N*-morpholino]ethanesulfonic acid) buffer (pH 6.1; 10 mM) containing cinidon-ethyl which was added in acetone + water (1 + 1 by volume; 5 ml litre^{-1} final concentration of acetone). The same concentration of acetone applied alone had no adverse effect. Incubation was in continuous light (55 μmol m^{-2}s^{-1}, 400–750 nm, Osram universal white neon tubes) at 25 °C for 24 h. The filter papers with leaf discs were then rolled cylindrically, placed in plastic tubes (13 mm diameter, 65 mm height) and sealed with rubber caps. After further incubation in light at 25 °C for 5 h, ethylene and ethane formation were quantified by gas chromatography.[10] The fresh weight of the leaf discs was measured subsequently. In a parallel experiment, leaf discs were incubated and parameters were measured in darkness at 25 °C. Afterwards, leaf discs of replicates were harvested, immediately frozen in solid carbon dioxide and powdered under liquid nitrogen. For determination of ACC, samples (100 mg, three replications) were extracted with ethanol + water (70 + 30 by volume). The ACC contents were assayed by converting them to ethylene, which was quantified by gas chromatography.[10,11]

For determination of Proto accumulation, leaf discs in Petri dishes were incubated in darkness at 25 °C for 24 h before exposure to light (105 μmol m^{-2}s^{-1}, 400–750 nm, Sylvania cool white neon tubes) for 2 h. Plant material was extracted and the quantitative determination of Proto was performed using a modification of the method described previously.[12,13] Samples

(100 mg) of powdered plant material were extracted with HPLC-grade methanol + 0.1 M ammonium hydroxide (9 + 1, by volume; 3 × 1.25 ml) under a dim, green light source. After centrifugation at 16 000*g* for 10 min, the supernatants were combined and evaporated to dryness at 36 °C with a rotary evaporator. The residue was dissolved in HPLC-grade methanol + 20 mM ammonium phosphate (9 + 1, by volume; pH 5.8; 1 ml), filtered through a 0.45-μm syringe filter and 100 μl of the sample was separated by reverse-phase HPLC on a LiChrosorb C_{18}-5 μm column (250 × 4 mm, Macherey-Nagel, Düren, Germany) using a gradient program from methanol + 20 mM ammonium phosphate (8 + 2, by volume; pH 5.8) to 100% methanol as described previously.[13] An aliquot of the fractions was removed and fluorescence was monitored for Proto using a Fluostar spectrophotometer (SLT, Crailsheim, Germany) with excitation and emission wavelength settings of 405 and 625 nm, respectively.

A commercial standard of Proto (Sigma Chemie, Deisenhofen, Germany) was used for calibration. Proto levels were expressed on a molar basis per gram fresh weight. The standard deviation of results obtained from parallel extractions of plant material was <10%.

2.4 Uptake, translocation and metabolism of [^{14}C]cinidon-ethyl

Plants at the second or third leaf stage were placed in plastic vials (25 mm diameter, 38 mm height; Greiner, Nürtingen, Germany) with 10 ml of half-strength Linsmaier–Skoog medium.[9] The vials were closed with plastic covers with slits into which the plants were fitted upright (one plant per vial, 10 replicates).

For the study of foliar uptake, 10 μl per plant of an aqueous solution containing [^{14}C]cinidon-ethyl (0.3 μg in 0.36 μl methanol, 1.85 KBq, *c* 100 000 dpm) and cinidon-ethyl EC (4.75 μg BAS 615 00H containing 0.95 μg AI.) was applied. The dose of cinidon-ethyl was approximately equivalent to a field rate of 50 g AI. ha^{-1} in 400 litres of carrier. Ten 1-μl droplets were applied with a microsyringe to the adaxial midsection of the primary leaf in wheat and *S nigrum* and to the surface of the four leaves of the first whorl in *G aparine*. Afterwards, the vials were placed in growth chambers under continuous light (530 μmol $m^{-2}s^{-1}$, 400–750 nm, Osram powerstar HQI-R 250 W/NDL and Osram krypton 100 W lamps) at 22 (±1) °C and 75 (±3)% relative humidity. In parallel experiments, plants were treated and incubated in darkness. The plants were harvested at 5, 16, 32 and 48 h after treatment. For the recovery and assessment of foliar deposits of the labelled herbicide on or adsorbed at the epicuticular wax layer of the leaf surfaces (non-absorbed radioactivity), cellulose acetate film stripping was used. This method has been shown to remove quantitatively the platelets of epicuticular wax that cover the intact leaf surface[14] and concomitantly the labelled herbicide, shortly after drying of the applied droplets (unpublished results). In order to differentiate between applied ^{14}C remaining on the surface of the epicuticular wax layer and that dissolved within it, treated leaf areas were cut from blades and intensively washed with water (plus 1 drop of Tween 20) for 10 min under shaking. The epicuticular wax layer of the leaf segments was then removed by cellulose acetate film stripping. Radioactivity was measured in the wash solutions (foliar deposit outside treated leaf) and in the cellulose acetate strips (foliar deposit adsorbed in epicuticular wax). For recovery of the radioactivity taken up in the leaf tissue (absorbed radioactivity), each plant was divided into treated leaf (after cellulose acetate film stripping), shoot part above treated leaf (apical shoot) and shoot part below treated leaf (basal shoot). After determination of fresh weights, plant parts were dried at 40 °C for three days, combusted in a biological materials oxidizer (Oxymat OX 300, Zinsser, Frankfurt, Germany) and the evolved [^{14}C]carbon dioxide was absorbed in Oxosolve C-400 LS cocktail (Zinsser). Radioactivity evolved from combusted plant parts and the cellulose acetate strips dissolved in acetone (LS cocktail Ultima Gold XR, Canberra-Packard, Frankfurt, Germany) was quantified by scintillation counting.

For study of root uptake, plants were placed hydroponically in 320-ml glass vessels (three to six plants per vessel, five replicates) in half-strength Linsmaier–Skoog medium[9] in growth chambers as described above. After one day of adaptation, 1 μM [^{14}C]cinidon-ethyl was added to the medium and plants were harvested at 32 h after treatment. Plants were removed from the medium and roots were carefully washed. The plants were then sectioned into root, basal shoot (1 cm shoot part above root in wheat, hypocotyl plus cotyledons in *S nigrum* and *G aparine*), and remaining shoot. Radioactivity in the plant parts was measured by liquid scintillation counting after combustion. In addition, uptake and translocation were studied by autoradiography. After treatment, the plants were dried and exposed to a film-like radiation image sensor (imaging plate, Raytest, Straubenhardt, Germany) for 72 h. The autoradiographs were quantified by densitometric scanning using the Bioimage analysis system (Analyzer BAS 1000, Fuji Photo Film Co, Tokyo, Japan).

For the study of metabolism, plants were placed in plastic vials and foliar-treated with 10 μl per plant of an aqueous solution containing [^{14}C]cinidon-ethyl (1.25 μg in 0.9 μl methanol, 7.71 kBq) and EC (6.25 μg) for 5 and 32 h as described above. The dose of cinidon-ethyl was equivalent to a rate of 50 g AI. ha^{-1} in 400 litres of carrier. Non-absorbed radioactivity was removed from the leaf surface using cellulose acetate stripping. Discs (0.5 cm in diameter) including the [^{14}C]cinidon-ethyl-treated areas were punched from the leaves of 15 plants (four replications) with a corkborer. The discs were immediately frozen in solid carbon dioxide and stored at −80 °C. Plant material (approximately 200 mg) was homoge-

nized in methanol + water (80 + 20 by volume; 5 ml) with a mortar and pestle and extracted three times for 1 h at 4 °C (four replicated extractions). After centrifugation, the combined supernatants were evaporated to dryness and redissolved in acetonitrile + water (60 + 40 by volume; 500 μl). Twenty microlitres of the sample were then separated by reverse-phase HPLC on a Spherisorb C_{18}-2 5 μm column (250 × 4 mm, Macherey-Nagel, Düren, Germany) using acetonitrile + water (60 + 40 by volume) as eluant. The sweep time was 29 min at a flow rate of 1 ml min^{-1}. Radioactivity of the fractions containing cinidon-ethyl (17.4 min) and its metabolites BAS 615M01 (5.2 min) and BAS 615M00 (15.5 min) was determined using a HPLC-coupled radioactivity monitor (LB 506 C, Berthold, Wildbad, Germany). As internal standard [^{14}C]cinidon-ethyl was added to the methanolic extract of the homogenized material. Recovery was above 80% in all cases after the extraction and HPLC procedure. The metabolites BAS 615M00 and BAS 615M01 were identified by their chromatographic retention and, in control experiments, by HPLC-electrospray-MS/MS (Sciex API III, Sciex, Concord, Ontario, Canada).

2.5 Plant toxicity studies

In connection with the foliar uptake of cinidon-ethyl, the time course of herbicide phytoxicity was investigated using the formation of ethylene and the reduction of leaf fresh weight as parameters. Plants at the second- or third-leaf stage in vermiculite substrate were foliar treated with cinidon-ethyl EC at 50 g AI ha^{-1} and incubated for 6, 21, 26 and 47 h under continuous light (530 μmol $m^{-2}s^{-1}$, 400–750 nm) at 22 °C and 75% relative humidity as described above. For determination of ethylene formation, treated primary leaves were dissected, placed in reagent tubes (15 mm diameter, 160 mm height; eight replicates) with 250 μl water and sealed with rubber caps. After incubation in light for 4 h at 25 °C, ethylene formation was quantified by gas chromatography. For determination of the effects on tissue fresh weight, discs (0.4 cm diameter, three discs per plant, six replicates) including the herbicide-treated areas were cut from blades of primary leaves with a corkborer and weighed. In parallel experiments, plants were treated for the indicated times and parameters were measured in darkness. In order to evaluate the herbicide selectivity between the weed species and wheat after foliar application of cinidon-ethyl EC, plants were cultivated hydroponically in 320-ml glass vessels as described above. They were sprayed uniformly on a turntable with 155 μl per vessel of aqueous emulsions containing cinidon-ethyl in increasing concentrations using an airbrush spray gun (Badger Profi 150, Revell, Bünde, Germany). At 32 h after treatment, fresh weights of shoots and roots were determined. In control treatments, aqueous solutions containing corresponding formulation concentrations but without cinidon-ethyl were applied, with no adverse effects.

2.6 Protox assays

Protox activity was extracted and assayed as described previously.[15–17] Plants at the second-leaf stage were homogenized in the cold with a Braun blender using a fresh weight-to-volume ratio of 1:4. Homogenization buffer consisted of Tris-HCl (50 mM; pH 7.3), sucrose (0.5 M), magnesium chloride, (1 mM), EDTA (1 mM) and bovine serum albumin (2 g $litre^{-1}$). After filtration through four layers of Miracloth, crude plastid preparations were obtained after centrifugation at 10 000 *g* for 5 min and resuspension in homogenization buffer before centrifugation at 150 *g* for 2 min to remove crude cell debris. The supernatant was centrifuged at 4000 *g* for 15 min and the pellet fraction was resuspended in 1 ml of a buffer containing Tris-HCl (50 mM; pH 7.3), EDTA (2 mM), leupeptin (2 μM), pepstatin (2 μM) and glycerol (200 ml $litre^{-1}$) and stored at −80 °C until use. Protein was determined in the enzyme extract by the method of Bradford[18] with bovine serum albumin as a standard. Protox activity was assayed fluorometrically by monitoring the rate of Proto formation from chemically reduced protoporphyrinogen IX under initial velocity conditions. The assay mixture consisted of Tris-HCl (100 mM; pH 7.3), EDTA (1 mM), dithiothreitol (2 mM), Tween 80 (0.3 g $litre^{-1}$), protoporphyrinogen IX (0.4 μM), and 0.1 to 0.2 mg extract protein (5 to 10 μl) in a total volume of 2.5 ml. The reaction was initiated by addition of the enzyme extract at 22 °C. Cinidon-ethyl and acifluorfen-methyl as a standard, prepared as stock solutions in acetone (below 1% final concentration in the assay), were added to the assay mixture in concentrations of 0.01 nM to 0.1 mM before incubation. Fluorescence was monitored directly from the assay mixture using a Perkin Elmer LS 50B spectrofluorometer with excitation at 405 nm and emission monitored at 633 nm. Non-enzymatic activity in the presence of heat-inactivated extract was negligible. Inhibition of enzyme activity induced by the herbicide was expressed as percentage inhibition relative to untreated controls. Molar concentrations of compounds required for 50% enzyme inhibition (I_{50} values) were calculated by fitting the values to the dose-response equation using non-linear regression analysis.

All experiments were repeated at least twice and proved to be reproducible. The results of a representative experiment are shown.

3 RESULTS AND DISCUSSION

3.1 Herbicidal selectivity

Spraying young plants of *G aparine* and *S nigrum* at the second-leaf stage with cinidon-ethyl caused rapid chlorophyll bleaching, desiccation, and necrosis of the green tissues. First phytotoxic symptoms of chlorophyll bleaching were evident 2–3 h after treat-

Table 1. Sensitivity of young plants of wheat, *Galium aparine* and *Solanum nigrum* to cinidon-ethyl (200 g litre^{-1} EC)

Plant species	Rate giving 30% reduction of shoot fresh weight (IC_{30}) (g AI ha^{-1})	SI[a]
Wheat	2100	
Galium aparine	4.3	488
Solanum nigrum	3.7	568

[a] The selectivity index (SI) was calculated as follows: SI = IC_{30} (wheat)/IC_{30} (weed).

ment if plants were placed in the light. Necrosis developed progressively, first on the youngest leaf blades and petioles and veins of older leaves. Complete necrosis of the entire shoot was observed two to three days after treatment with 50 g ha^{-1} cinidon-ethyl in the EC formulation. Visual symptoms of injury were identical to those induced by the Protox-inhibiting diphenyl ether herbicide acifluorfen-methyl (observations not shown). Plants treated and incubated in the dark did not develop damage (not shown). Local application of cinidon-ethyl in single droplets caused necrotic spots on leaves which were strictly localized to the application area. This suggests that cinidon-ethyl works primarily by contact action in sensitive plant species. In order to quantify the selectivity between the weed species *G aparine* and *S nigrum* and the tolerant wheat, plants were uniformly sprayed with increasing rates of EC formulation (0.1 g ha^{-1} to 2400 g ha^{-1} cinidon-ethyl) and incubated for 24 h under continuous light conditions. The doses of cinidon-ethyl required for 30% reduction of shoot fresh weight (IC_{30} values) were calculated from linear regression equations (Table 1). As shown in Table 1, the selectivity index of cinidon-ethyl between wheat and *S nigrum* or *G aparine* (SI) was approximately 500. This corresponds with results obtained after post-emergent treatment of young plants grown under greenhouse conditions (K-O Westphalen, H Walter, BASF Agricultural Center Limburgerhof, pers comm).

3.2 Mode of action

By virtue of structural affinity, light requirement for phytotoxicity and the symptomology of plant damage, we hypothesized that cinidon-ethyl belongs to the group of Protox-inhibiting, peroxidizing herbicides. Since the succeeding series of events induced by these herbicides includes inhibition of Protox and accumulation of Proto, followed by photodynamic membrane lipid peroxidation, formation of ethylene, and tissue necrotization,[1–3] the influences of cinidon-ethyl on these processes were investigated. As shown in Table 2, Protox activities assayed from crude plastid preparations of *S nigrum*, *G aparine* and wheat were inhibited *in vitro* by very low concentrations of cinidon-ethyl. The I_{50} concentrations for Protox inhibition were around 1 nM cinidon-ethyl (Table 2). No significant differences in the sensitivity of *in vitro* Protox activity to cinidon-ethyl between the weed species and wheat were found. In comparison to the known Protox inhibitor acifluorfen-methyl,[7] cinidon-ethyl showed similar enzyme-inhibiting activity (Table 2). The blockage of Protox prevents the normal enzymatic conversion of protoporphyrinogen IX which diffuses into the cytoplasm where it leads to Proto accumulation after oxidation.[1,2,5,6] When leaf discs of *S nigrum*, *G aparine* and wheat were treated with cinidon-ethyl for 24 h in darkness before exposure for 2 h in light, a concentration-dependent increase in Proto levels was found in the weed species (Fig 2). At 1 μM cinidon-ethyl, Proto levels increased approximately 4-fold and 2-fold in *Solanum* and *Galium*, respectively, and reached approximately 10 times the levels of untreated controls in both weed species at 100 μM (Fig 2). Effects were detectable from a concentration of 100 nM. In the light, Proto-mediated

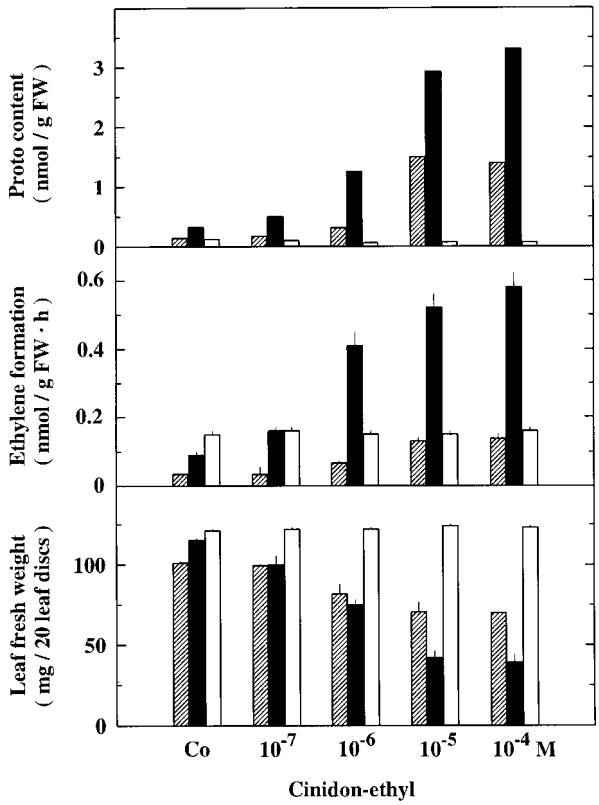

Figure 2. Influence of cinidon-ethyl on Proto accumulation, ethylene formation and fresh weight of leaf discs from (▨) *Galium aparine*, (■) *Solanum nigrum* and (□) wheat. Vertical bars represent SE of the mean.

Table 2. Effects of cinidon-ethyl on Protox activity extracted and assayed from wheat, *Galium aparine* and *Solanum nigrum*

	Concentrations required for 50% inhibition (I_{50}) (nM)	
Plant species	Cinidon-ethyl	Acifluorfen-methyl
Wheat	1.30	1.10
Galium aparine	0.75	0.87
Solanum nigrum	0.94	0.49

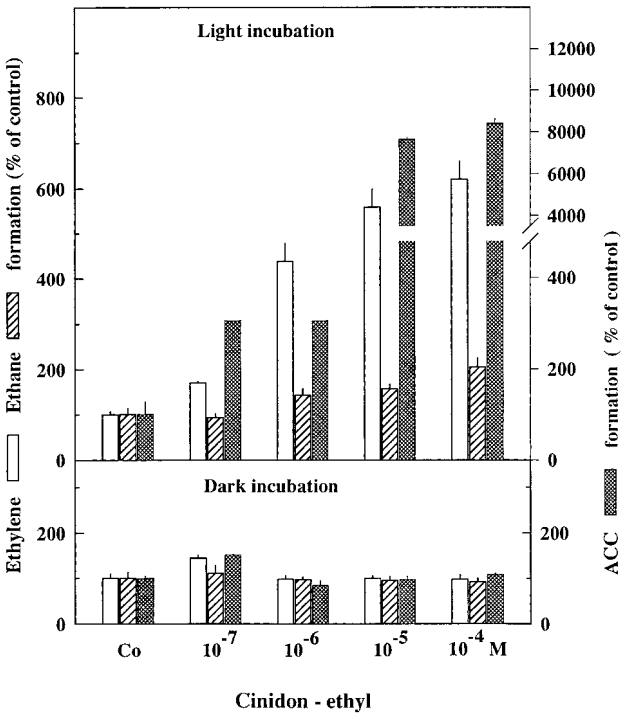

Figure 3. Influence of cinidon-ethyl on (□) ethylene and (▨) ethane formation and (▩) ACC levels in leaf discs of *Solanum nigrum* after 24 h of treatment in light and darkness. Vertical bars represent SE of the mean.

generation of activated oxygen causes the formation of stress ethylene, membrane lipid peroxidation accompanied by ethane evolution and tissue desiccation.[1] Light-dependent stimulation of ethylene formation and reduction in leaf fresh weight were induced by cinidon-ethyl only in the weed species (Figs 2, 7). At 100 μM cinidon-ethyl, ethylene formation increased approximately 6-fold and 3-fold in *S nigrum* and *G aparine*, respectively (Fig 2). The dose-response of Proto accumulation correlated with the increase in ethylene formation, accumulation of ACC, the direct precursor of ethylene in its biosynthesis,[19] and with the reduction in leaf fresh weight (Figs 2, 3). At 100 μM cinidon-ethyl in *S nigrum*, levels of ethylene and ACC increased 6-fold and 80-fold, relative to the control, respectively (Fig 3). This suggests that ethylene production is stimulated by cinidon-ethyl through stress-activating ACC synthesis.[20] In contrast, cinidon-ethyl did not induce Proto accumulation and subsequent effects in wheat (Fig 2). As an end-product of lipid peroxidation, ethane evolution also increased, dependent on the herbicide concentration (Fig 3). In *S nigrum* leaf discs, a maximum of twice the level of control was reached (Fig 3). Cinidon-ethyl did not change ethylene and ethane evolution, ACC levels and the leaf fresh weight of plant tissue incubated in the dark (Fig 3). It is concluded that cinidon-ethyl is a Protox-inhibiting herbicide with peroxidative mode of action. The selectivity of cinidon-ethyl in wheat appears not to be based on a lower sensitivity to the herbicide of the enzymatic target.

3.3 Uptake and distribution of [^{14}C]cinidon-ethyl

After foliar treatment of *G aparine*, *S nigrum* and wheat plants with [^{14}C]cinidon-ethyl EC formulation equivalent to a field rate of 50 g AI ha^{-1}, no significant differences between absorption and translocation of the [^{14}C]-herbicide were found (Figs 4, 5). Approximately 15% of the [^{14}C]-compound was taken up within 32 h under standard conditions (continuous light, 22 °C, 75% relative humidity; Fig 5). Using cellulose acetate film stripping, nearly 85% of applied ^{14}C was shown to remain on or adsorbed within the epicuticular wax layer of the leaf surfaces (Fig 5). For assessment of the distribution between both deposits, treated leaf areas were washed with water before cellulose acetate stripping. Already 5 h after herbicide application, only *c*30%, 40% and 20% of applied radioactivity could be removed by the washing procedure in *G aparine*, *S nigrum* and in wheat,

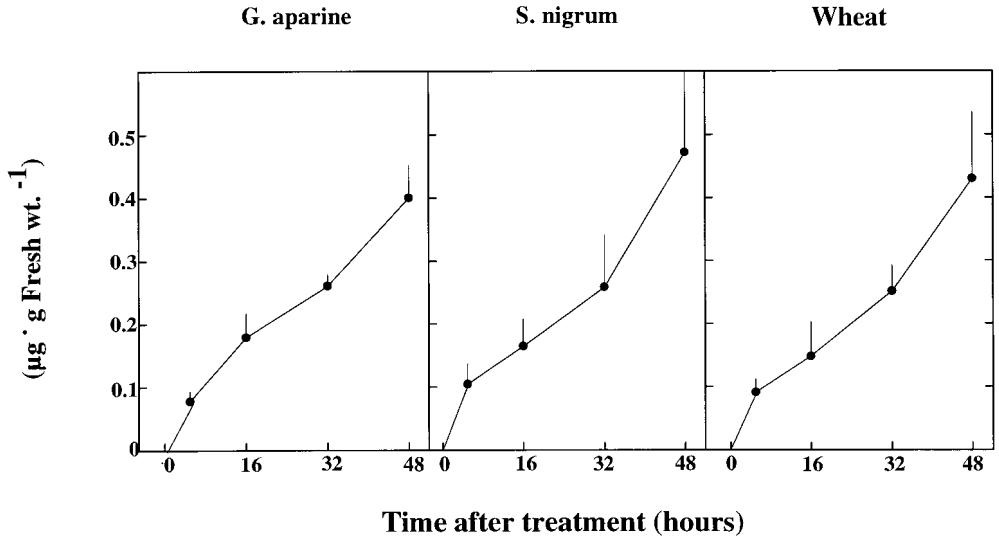

Figure 4. Time course of foliar absorption of [^{14}C]cinidon-ethyl in *Galium aparine*, *Solanum nigrum* and wheat. Data are averages of 10 replicate plants per point. Vertical bars represent SE of the mean.

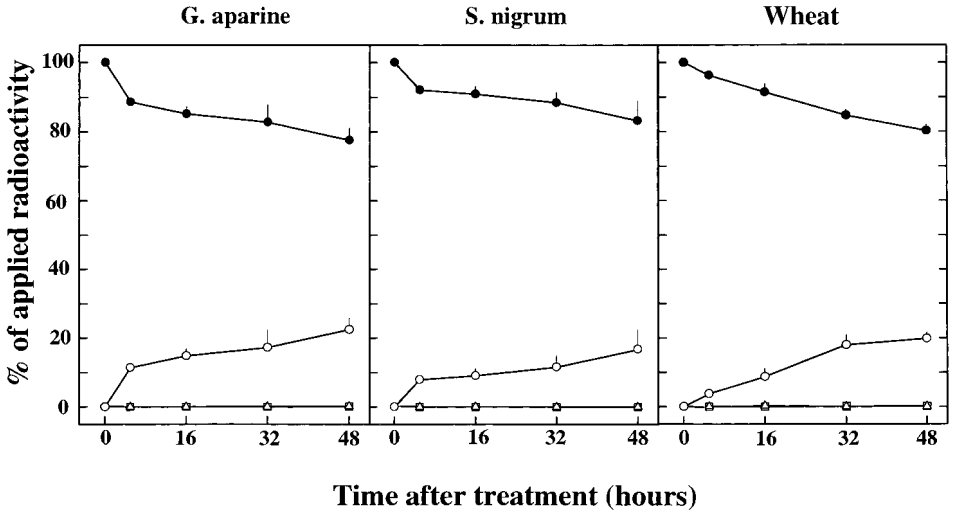

Figure 5. Time course of foliar absorption and translocation of [^{14}C]cinidon-ethyl in *Galium aparine, Solanum nigrum* and wheat. (●) Radioactivity on or adsorbed at the epicuticular wax layer of the leaf surfaces, (○) radioactivity inside treated leaves, (□) radioactivity translocated to the apical shoot, (△) radioactivity translocated to the basal shoot. Data are averages of 10 replicate plants per point. Vertical bars represent SE of the mean.

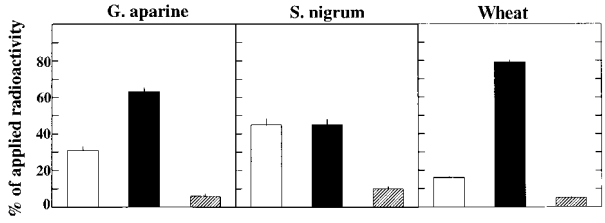

Figure 6. Distribution of foliar deposits of [^{14}C]cinidon-ethyl in *Galium aparine, Solanum nigrum* and wheat. (□) Foliar deposit on outside of treated leaf, (■) foliar deposit adsorbed in epicuticular wax and in (▨) absorbed radioactivity. Vertical bars represent SE of the mean.

respectively (Fig 6). The main portions of ^{14}C remained associated with the epicuticular wax layers and were only subsequently removed by cellulose acetate film stripping (Fig 6). This explains the high rainfastness of the activity of cinidon-ethyl under practical conditions (O Schmidt, BASF Agricultural Center Limburgerhof, pers comm). It also indicates that the lipophilic herbicide molecules are rapidly adsorbed by the epicuticular wax layer of the leaf surface and subsequently penetrate into the leaf more slowly. When cinidon-ethyl was applied to stem parts (first internode) of *G aparine* plants, the uptake was approximately 2-fold higher than that after leaf treatment (data not shown). Calculated from combustion data, less than 0.3% of absorbed ^{14}C had been translocated beyond treated leaves to the apical or basal shoot parts (Fig 5). In the weed species, the increase in herbicide uptake correlated with the induction of phytotoxic effects (Figs 4, 7). The herbicide-treated leaf areas showed a decreased fresh

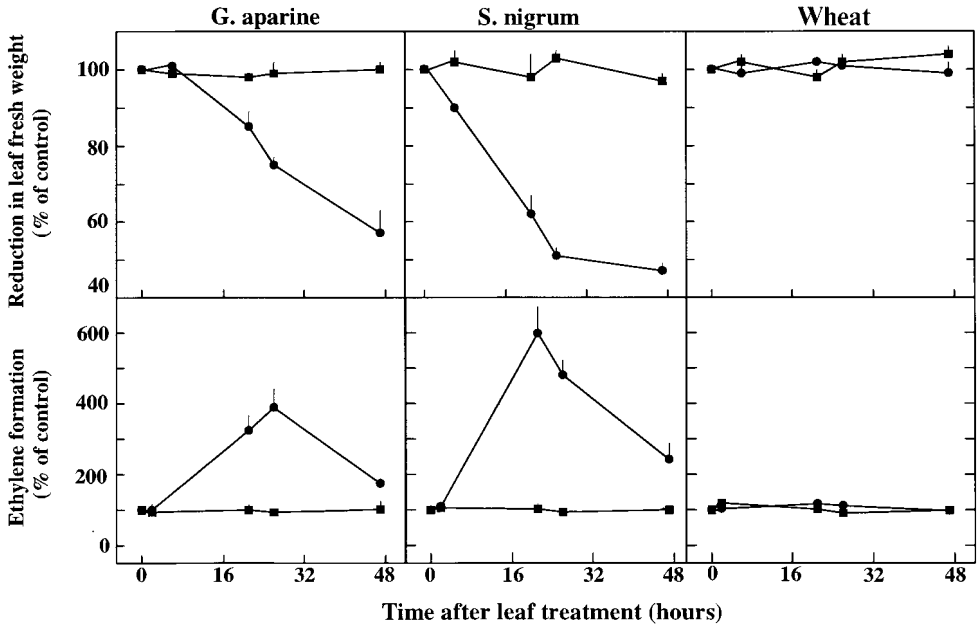

Figure 7. Time course of the effects of foliar-applied cinidon-ethyl on ethylene formation and fresh weight of leaf tissue in *Galium aparine, Solanum nigrum* and wheat (●) in light and (■) in darkness. Vertical bars represent SE of the mean.

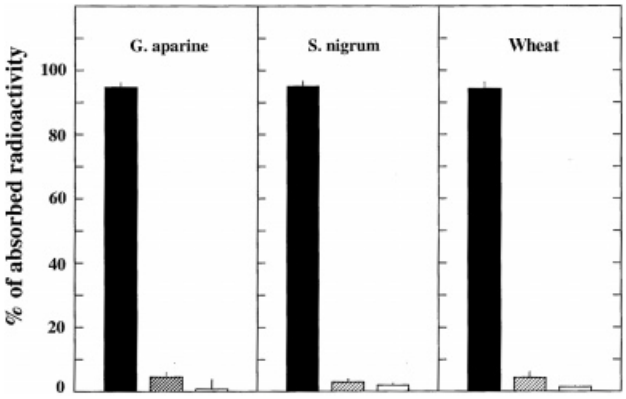

Figure 8. Root absorption and translocation of [^{14}C]cinidon-ethyl in *Galium aparine, Solanum nigrum* and wheat. Radioactivity (■) in root (▨) in basal shoot and (□) in remaining shoot. Vertical bars represent SE of the mean.

weight and a transient rise in ethylene formation (Fig 7). After foliar treatment of *S nigrum*, *G aparine* and wheat plants with [^{14}C]cinidon-ethyl EC at 50 g AI ha^{-1}, the influence of darkness on herbicide absorption was studied. No significant differences between uptake of the herbicide in the light and darkness were found 32 h after application (data not shown). After treatment of *S nigrum*, *G aparine*, and wheat plants with 1 μM [^{14}C]-cinidon-ethyl hydroponically, the bulk of the absorbed radioactivity was retained in the roots during an incubation period of 32 h (Fig 8). Approximately 5% of the root-absorbed ^{14}C was translocated to the basal shoot parts of the plants and only trace amounts of radioactivity were found in the upper shoot parts. Consequently, herbicidal effects were not observed in the plants. The limited translocation of cinidon-ethyl in the plant was confirmed by autoradiography of *S nigrum*, *G aparine* and wheat plants after foliar and root exposure to [^{14}C]cinidon-ethyl for 32 h (data not shown). Hence, cinidon-ethyl is effective through contact action in the green tissue of sensitive weed species. The selective action of cinidon-ethyl is not based upon differences in uptake or translocation of the herbicide.

3.4 Metabolism of [^{14}C]cinidon-ethyl

Experimental procedures with *S nigrum*, *G aparine* and wheat plants were similar to those used for investigation of foliar uptake and translocation. Plants were treated with [^{14}C]cinidon-ethyl EC at 50 g AI ha^{-1} and the non-absorbed radioactivity was removed from the leaf surface after 32 h. Metabolism was then studied in the herbicide-treated leaf. After extraction and HPLC-radiocounting, cinidon-ethyl and its initial metabolites, the *E*-isomer of the parent (BAS 615M00), and the acid (BAS 615M01) generated by the cleavage of the ethyl ester bond and its isomers were identified. When applied to plants postemergently (K-O Westphalen, H Walter, BASF Agricultural Center, Limburgerhof, pers comm), these metabolites showed approximately 10 times lower herbicidal activity than the parent compound. In accordance, using Protox enzyme isolated from maize, I_{50} values of the *E*-isomer of cinidon-ethyl and of the free acid derivative were 11 nM and 4.1 nM, respectively. The radioactivity found in the peaks of subsequent metabolites and conjugates of metabolites with endogenous plant substances (dissolved bound residues, F Mayer, BASF Agricultural Center Limburgerhof, pers comm) was totalled and is indicated as 'others' in Fig 9. Distinct differences were found in the metabolism of cinidon-ethyl in the plant species treated for 5 h and 32 h (Fig 9). Wheat had metabolized more of the parent herbicide than did either of the weeds. After 32 h, in the leaf tissues of wheat, *S nigrum* and *G aparine* approximately 12, 36, and 47% of the absorbed radioactivity, respectively, was found as unchanged herbicide or as the first, biologically less active metabolites (Fig 9). Only 4% of the parent molecule remained in the treated tissue of wheat after 32 h exposure, while 18% and 33% remained in *S nigrum* and *G aparine*, respectively (Fig 9). Approxi-

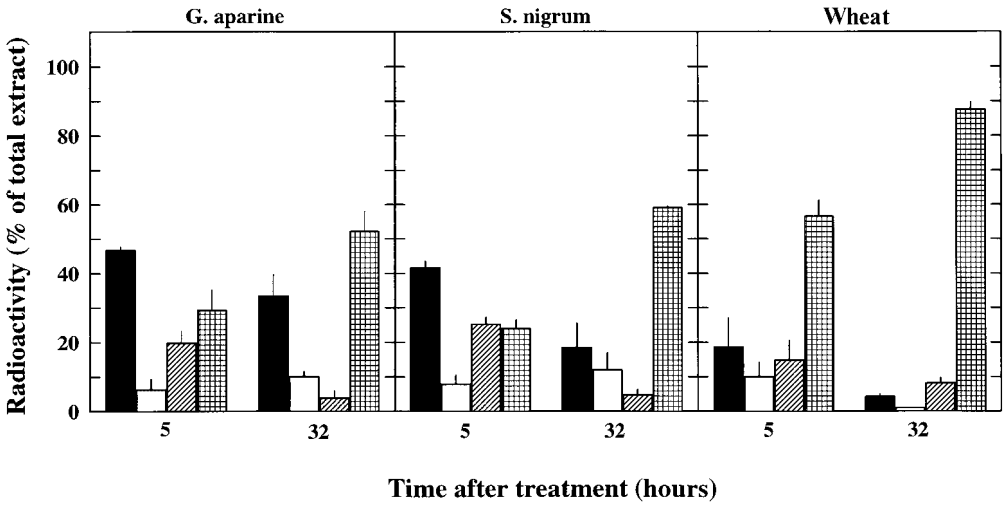

Figure 9. Metabolism of [^{14}C]cinidon-ethyl in leaf tissue of *Galium aparine, Solanum nigrum* and wheat. (■) Cinidon-ethyl, (□) E-isomer of cinidon-ethyl (BAS 615M00), (▨) free acid (BAS 615M01) and isomers, (▦) other metabolites. Vertical bars represent SE of the mean.

mately 88, 59, and 52% of the radioactivity was found as products of advanced metabolization of cinidon-ethyl ('others' in Fig 9) in wheat, *S nigrum*, and *G aparine*, respectively. At 5h after treatment, approximately 18, 42, and 47% of the absorbed radioactivity was found as unchanged herbicide in wheat, *S nigrum*, and *G aparine*, respectively (Fig 9). It is suggested that a more rapid metabolism combined with moderate leaf absorption are the primary factors that contribute to the tolerance of wheat to cinidon-ethyl.

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REFERENCES

1 Matringe M, Camadro J-M and Brouillet N, Protoporphyrinogen oxidase, the molecular target site for peroxidizing herbicides. *Proc Brighton Crop Protection Conf – Weeds* pp 703–711 (1993).

2 Böger P and Wakabayashi K, Peroxidizing herbicides (I): mechanism of action. *Z. Naturforsch* **50c**:159–166 (1995).

3 Dayan FE and Duke SO, Overview of protoporphyrinogen oxidase-inhibiting herbicides. *Proc Brighton Crop Protection Conf – Weeds* pp 83–92 (1997).

4 Beale SI and Weinstein JD, Tetrapyrrole metabolism in photosynthetic organisms, in *Biosynthesis of Heme and Chlorophyll*, ed by Dailey HA, McGraw-Hill, New York. pp 287–391 (1990).

5 Jacobs JM and Jacobs NJ, Porphyrin accumulation and export by isolated barley (*Hordeum vulgare*) plastids. *Plant Physiol* **101**:1181–1187 (1993).

6 Retzlaff K and Böger P, An endoplasmic reticulum plant enzyme has protoporphyrinogen IX oxidase activity. *Pest Biochem Physiol* **54**:105–114 (1996).

7 Anderson RJ, Norris AE and Hess FD, Synthetic organic chemicals that act through the porphyrin pathway, in *Porphyrin Pesticides. Chemistry, Toxicology, and Pharmaceutical Applications*, ed by Duke SO and Rebeiz CA, *ACS Symposium* Series **559**:pp 18–33 (1994).

8 Nuyken W, Grossmann K, Plath P, Vantieghem H and Westphalen K-O, *New herbicidal N-phenyltetrahydrophthalimids*. Poster Abstract 1C-023, 9th International Congress Pesticide Chemistry, London UK (1998).

9 Linsmaier EM and Skoog F, Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* **18**:100–127 (1964).

10 Grossmann K, Häuser C, Sauerbrey E, Fritsch HJ, Schmidt O and Jung J, Plant growth retardants as inhibitors of ethylene production. *J Plant Physiol* **134**:538–543 (1989).

11 Lizada MCC and Yang SF, A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. *Anal Biochem* **100**:140–145 (1979).

12 Nandihalli UB, Sherman TD, Duke MV, Fisher JD, Musco VA, Becerril JM and Duke SO, Correlation of protoporphyrinogen oxidase inhibition by *o*-phenyl pyrrolidino- and piperidinocarbamates with their herbicidal effects. *Pestic Sci* **35**:227–235 (1992).

13 Matsumoto H, Tanida Y and Ishizuka K, Porphyrin intermediate involved in herbicidal action of δ-aminolevulinic acid on duckweed (*Lemna paucicostata* Hegelm). *Pest Biochem Physiol* **48**:214–221 (1994).

14 Silcox D and Holloway PJ, A simple method for the removal and assessment of foliar deposits of agrochemicals using cellulose acetate stripping film. *Asp Appl. Biol* 11, *Biochemical and Biophysical Techniques in Herbicide Research*. pp 13–17 (1986).

15 Jacobs NJ and Jacobs JM, Assay for enzymatic protoporphyrinogen oxidation, a late step in heme synthesis. *Enzyme* **28**:206–216 (1982).

16 Labbe P, Camadro JM and Chambon H, Fluorimetric assays for coproporphyrinogen oxidase and protoporphyrinogen oxidase. *Anal Biochem* **149**:248–260 (1985).

17 Nicolaus B, Sandmann G and Böger P, Inhibition of protoporphyrinogen oxidase from maize, in *Target Assays for Modern Herbicides and Related Phytotoxic Compounds*, ed by Böger P and Sandmann G, Lewis Publ Boca Raton, FL. pp 35–41 (1993).

18 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).

19 Yang SF and Hoffman NE, Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev Plant Physiol* **35**:155–189 (1984).

20 Abdallah MM, Bayer DE and Elmore CL, Effects of light and ethylene on the herbicidal action of acifluorfen. *Pest Biochem Physiol* **42**:271–278 (1992).